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13. ABSTRACT ***(Maximum 200 Words)***
The search of chemopreventive strategies for breast cancer is imperative. It is vital to identify critical early events that can increase the risk that a normal epithelial mammary cell may be transformed into a breast cancer cell. Exposure to estrogen, already, recognized as a predisposing event for breast cancer, formed the rationale for preventive trials based on an antiestrogen compound (tamoxifen). This proposal explores the mechanism of another likely predisposing event for breast cancer, the loss of expression of the retinoic acid receptor beta (RAR beta), a nuclear receptor that responds to derivatives of vitamin A. Loss of expression of RAR beta was reported in breast cancer, as well as in other epithelial cancers. Our IDEA project aims to understand the mechanisms involved in RAR beta loss, in order to devise strategies not only to reverse this loss, but also to prevent it. In the first year of investigation, we found that a mechanism of transcriptional silencing of genes called hypermethylation is an important factor of irreversible RAR beta loss in breast cancer cells. In the second year we tested in depth the epigenetic mechanisms (deacetylation of P2) leading to downregulation and loss of RAR beta expression and development of RA resistance. This paves the road to a) treatment of RA-resistant breast cancer and 2) chemoprevention of breast cancer by maintaining an appropriate activity of the RAR beta P2 promoter to deter the occurrence of epigenetic events responsible for RAR beta silencing and RA-resistance.

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RAR BETA METHYLATION AND LOSS OF RAR BETA EXPRESSION IN BREAST CANCER (PI : SACCHI N)

INTRODUCTION

This proposal explores the mechanism of another likely predisposing event for breast cancer, the loss of expression of the retinoic acid receptor beta (RAR beta), a nuclear receptor that responds to derivatives of vitamin A, including retinoic acid (RA) (Chambon, 1996). Loss of expression of RAR beta was reported in both breast cancer cell lines and primary breast tumors (Swisshelm et al., 1994; Seewaldt et al., 1995; Li et al., 1995; Tsou et al., 1998; Widshwendtner et al., 1997; Xu et al., 1997). The expression can be reinduced in some breast cancer lines by RA treatment, whereas in other breast cancer cell lines RAR beta expression cannot be reversed (Liu et al., 1997). These breast cancer cell lines can be considered a model of primary breast tumors resistant to RA-differentiation therapy (Baust et al., 1996).

Our project aims to understand the causes of RAR beta loss and devise strategies both to revert this loss and possibly to prevent it. We originally proposed to investigate:

1) whether a mechanism of transcriptional silencing of genes called hypermethylation is a factor of the irreversible RAR beta loss in breast cancer (Task 1);

2) whether RAR beta expression can be restored by overriding methylation either altering the chromatin state or by demethylating the RAR beta 2 promoter (Task 1);

3) whether methylation of RAR beta can be prevented by preventing the inactivity of RAR beta 2 promoter (Task 2).

We report that indeed hypermethylation is a factor in RAR beta loss and that RAR beta loss can be restored by chromatin remodeling drugs. The proposed course aims at understanding how hypermethylation can be prevented, in order to prevent RAR beta loss and its consequences.

BODY

In the approved Statement of Work (SOW) we originally proposed to perform two Tasks, 1 and 2.

Task 1. To determine the expression of the RAR beta gene and the methylation status of the RAR beta 2 promoter region in human epithelial mammary cells (HMEC) and breast cancer cell lines and analyze the demethylating effect of 5-Aza-2'-deoxycytidine (5 -Aza-CdR) on re- activation of RAR beta (months 1-12).

We were able to successfully complete all the experiments planned to accomplish Task 1. First, we tested the hypothesis that loss of RAR beta activity occurs as a result of multiple factors, including aberrant methylation of the regulatory region of RAR beta, an epigenetic change which can pattern the RAR beta chromatin state and influence gene activity. Using methylation-specific PCR (MSP), we found hypermethylation in the promoter P2 of the RAR beta gene in a significant proportion of both breast cancer cell lines and primary breast tumors (See Appendix 1, paper by Sirchia et al., Oncogene, 2000).

Second, we observed that the chromatin changes induced by methylation at the level of RAR beta promoter can be reversed, with subsequent reexpression of the gene, and

restoration of RA sensitivity. We showed that treatment of breast cancer cell lines with a methylated P2 promoter, by means of the DNA methyltransferase inhibitor 5-Aza-2'-deoxycytidine (5-Aza-CdR), led to demethylation of the RAR beta 2 promoter and expression of RAR beta indicating that DNA methylation is at least one factor, contributing to RAR beta inactivity (see Appendix 1, Sirchia et al., Oncogene, 2000). We now know, that DNA methylation causes an altered acetylation by recruiting the MeCP2/Sin3A/HDAC complexes (Nan et al., 1998; Wade et al., 1998). For this reason, we tested whether RAR beta activity could be restored by simply altering the repressive chromatin state at the level of RAR beta 2 promoter. We found that the more stable repressive RAR beta 2 status in the RA-resistant MDA-MB-231 cell line can be alleviated by the HDAC inhibitor, trichostatin A (TSA), with restoration of RA-induced RAR beta transcription. In Appendix 1, we enclose two manuscripts (Sirchia et al., 2000; Virmani et al., 2000) and three abstracts summarizing the results of: a) our breast cancer study and b) a collaborative study on lung cancer with Dr. A. Virmani at the Hamon Center for Therapeutic Oncology Research in Dallas, TX.
Dr. Virmani extended the experimental approach devised for our breast cancer study to demonstrate that RAR beta hypermethylation results in RAR beta loss in a high proportion of breast cancers. Altogether these results show that hypermethylation associated with RAR beta loss is not limited to breast cancer alone and broaden the scope of our preventive study.

Hypermethylation of RAR beta gene in breast cancer may be utilized for several clinical applications including:
1) *Early diagnosis of breast cancer.* Detection of methylation by methylation specific PCR (MSP) (Herman et al., 1996) is very sensitive. Each tumor type, including breast cancer has specific methylated genes that can be exploited as diagnostic and early detection markers (Belinski et al., 1998). A methylated gene in order to be useful as an early detection marker must fulfil a number of criteria, including: a) to be present in at least 30% of breast cancers; b) to be unmethylated in peripheral blood lymphocytes and normal stroma, as well as in stroma and support tissue adjacent to the breast tumor; c) should be methylated in early disease stage. Each of these criteria is met by RAR beta that can be included in a panel of hypermethylated markers for breast cancer detection (Belinski et al, 1998). Currently, a panel of hypermethylated markers including RAR beta is used at JHU for early detection of tumor cells on breast ductal cell samples obtained by the procedure of ductal lavage technique.

2) *Detection of RA-resistant tumors.* It has been reported that a fraction of breast cancers are refractory to RA-differentiation therapy (Baust et al., 1996). Now we know that breast cancer with hypermethylated RAR beta are RA-resistant. Detection of the fraction of breast cancer that are methylated can identify the breast cancers that are unlikely to respond to RA-differentiation therapy. This may assist in the selection of patients that may benefit from therapeutic protocols including RA.

3) *Improving the treatment of RA-resistant breast cancer.* We have shown that by using RA in combination with chromatin-remodeling drugs, like the histone deacetylase inhibitor Trichostatin A, is possible to restore RA-sensitivity in breast cancer cells in

vitro. This suggests that treatment of RA-resistant tumors might be improved by combining RA to histone deacetylase inhibitors. This drug combination has already been used in the treatment of leukemia (Warrell et al., 1998) and may be exploited to overcome the hurdle of RA-resistance in breast cancer.

Task2. To attempt the induction of the methylation status of the RAR beta P2 promoter by inhibiting RAR beta activity (months 13-24)

Breast cancer could be prevented if we could identify ways to prevent the occurrence of key molecular changes leading to transformation of mammary epithelial cells. Loss of RAR beta seems to be a key change. Recently, it has been discovered that loss of RAR beta causes the down regulation of expression of the intracellular adhesion molecule-1 gene (ICAM) and the MHC class I heavy chain . This fact strongly indicates that RAR beta deficient tumor cells may escape immunological surveillance (Toulouse et al., 2000). In light of this finding, it is clear that prevention of irreversible RAR beta gene inactivity may be very relevant.

In order to prevent RAR beta loss we need to understand the series of events leading to RAR beta gene inactivity. We first hypothesized that DNA-methylation is secondary to prolonged inactivity of the RAR beta P2 promoter. RAR beta P2 is regulated in the presence of RA by its receptors RAR alpha and RAR beta itself (Gudas et al., 1994; Chambon, 1996). In the presence of RA the receptors activate RAR beta P2 by tethering protein complexes endowed with histone acetylase activity (HAT) and, in the absence of RA, of protein complexes with histone deacetylase activity (HDAC) (Chambon, 1996). Histone deacetylation of P2 may predispose to DNA methylation, a condition that might further attract histone deacetylation mediated by the MeCP2/Sin3A/HDAC corepressor complex (Nan et al., 1998; Wade et al., 1998; Razin, 1998; Ng and Bird, 1999; Jones and Wolffe, 1999). The idea that gene inactivity invites"de novo methylation was first proposed by Bird, back in 1986. This suggestion was further refined after the discovery of the mechanistic link between DNA methylation and chromatin conformation. Ng and Bird recently proposed that: "DNA methyltransferase –either independently or assisted by accessory proteins –may be capable of reading the histone acetylation pattern on the chromatin and its de novo methyltransferase activity can respond differentially to different states of chromatin modification. In this case, deacetylated chromatin would provoke de novo methylation. This self-reinforcing mechanism, supported by DNA methylation and histone deacetylation, could provide a stable state of inactive chromatin, unless overcome by other mechanisms".

The RAR beta P2 promoter can provide an ideal system to test this as well as our hypothesis.

Methods

We performed a mechanistic study to understand the link between RAR β P2 activity and the epigenetic events (methylation/acetylation of H3 and H4) in unmethylated breast cancer cell lines (T47D) where RAR β is partially inactive, that is down-regulated and in cancer cell lines (MCF7 and MDA-MB-231) where RAR β is silenced, that is irreversibly inactivated in the absence and presence of RAR β agonist, RA. We also studied the reactivation of RAR β P2 using both demethylating agents (5-Aza-CDR) and HDACI like the phenyl butyrate and Trichostatin A (TSA) Cells were tested for RAR beta transcription(by means of RT-PCR). In parallel we tested for changes in the histone acetylation status of the chromatin embedding the RAR beta promoter 2 (by Chromatin Immunoprecipitation analysis) and hypermethylation of RAR beta (by MSP analysis). ChIP and MSP analyses were performed as previously described (Keshet ET al., 1986; Hebbes ET al., 1994; Eden ET al., 1998).

We found a direct correlation between induction of RAR beta inactivity and occurrence of changes in the acetylation status of RAR beta P2. These studies indicate a precise need to maintain the chromatin of P2 relaxed in order for the ligand (RA) to perform its action. Moreover indicate that a specific reacetylation at P2 restores RAR β transcription even when P2 is methylated.
These data unequivocally show that the occurrence of RAR beta downregulation/silencing not only can be reversed, thus representing a target for molecular therapy of breast cancer, but it could be prevented by devising strategies to keep RAR beta active (Sirchia et al., manuscript in preparation).

Strategies to maintain RAR beta P2 transcriptional activity warrant additional investigations. One strategy could be to maintain supraphysiological concentration of RA in epithelial cells (Minna and Mangeldorf, 1997). RA has been already explored as a chemoprevention agent. However, despite the potential, the use of RA has been limited in the chemopreventive setting by its toxicity. Several new retinoids are being synthesized and tested in vitro and in preclinical studies. Another strategy would be to increase the levels of endogenous RA by inhibiting the cytochrome P450 –mediated catabolism of RA using a novel class of agents, the retinoic acid metabolism blocking agents (RAMBAs). Such as Liarazole, already used in clinical trials (Miller, 1998).

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration that the RAR β promoter 2 gene is hypermethylated in breast cancer cell lines and approximately one third of primary breast cancers
- Evidence that hypermethylated RAR β meets the criteria of an early detection marker and can then be used in combination with other methylated markers for molecular analysis of ductal breast cancer cells in ductal lavage fluid (Evron et al., 2001)
- Evidence that hypermethylated RAR β not only is a marker of breast cancer, but also a "predictor" marker of RA-resistance (Sirchia et al., manuscript in preparation)
- Evidence that RAR β activity can be enhanced/restored by inducing an adequate level of acetylation at the P2 promoter combining histone deacetylase inhibitors (phenyl butyrate (PB) and trichostatin A (TSA) and RA
- Evidence that reacetylation of the promoter P2 from unmethylated/methylated RAR β P2 promoter and RAR β reactivation is associated with tumor growth inhibition

REPORTABLE OUTCOMES

One published paper

Evron E., Dooley WC, Umbricht CB, Rosenthal D, **Sacchi N**, Gabrielson E, Soito AB,Hung DT,Ljung BM, Davidson ME, Sukumar S
Detection of breast cancer cells in ductal lavage fluid by methylation-specific PCR TheLancet 357, 1335-36, 2001

In preparation

Silvia Sirchia, Roberto Pili, Salvatore Toma, Elena Sironi, Giulia Somenzi, Riccardo Ghidoni, Mingqiang Ren, Guido Nicolo', Nicoletta Sacchi
Molecular targeting of RAR Beta As A Strategy To Overcome Epigenetic Retinoic Acid -Resistance In Breast Cancer

Four abstracts

1) N. Sacchi, S. Sirchia, E. Sironi, S. Sukumar
Chromatin remodeling at RAR β Promoter is associated with RA-resistance in Breast Cancer
Proceedings 92th AACR Meeting, New Orleans, April 2001

2) Evron E, Dooley W, Umbricht C, Rosenthal D, **Sacchi N**, Davidson N, Sukumar S
Molecular detection of breast cancer cells in ductal lavage fluid (DLF) using methylation specific PCR
Proceedings ASCO, 2001

3) S.Sirchia, E.Sironi, G. Somenzi, S.Pozzi and **N. Sacchi**
RAR β gene re-expression therapy for breast cancer
Joint International Congress on APL and Differentiation Therapy, Rome,October 4-7, 2001

4) S.Sirchia, E.Sironi, G. Somenzi, R. Pili, S. Pozzi and **N. Sacchi**
Targeting epigenetic changes associated with retinoid resistance: a novel strategy to treat breast cancer
Am J Hum Genet Vol 69 (Supplement) A 151, p. 205, 2001

CONCLUSIONS

At this time we provide evidence that DNA-methylation at RAR beta P2 promoter in breast cancer cells is an important factor affecting RAR beta transcription.
We obtained preliminary evidence that demethylation and histone acetylation of RAR

beta P2 promoter are related, but distinct mechanisms for destabilizing chromatin and reactivating transcription in methylated cell lines.

We propose to continue this study to analyze whether prolonged (forced) inactivity of RAR beta is a prerequisite for the occurrence of hypermethylation/deacetylation of the P2 promoter and irreversible RAR beta loss.

This work has diagnostic, therapeutic and preventive implications.

Diagnostic implications: Knowledge of RAR beta P2 methylation state of primary breast cancers is useful to identify tumors that are likely to respond to RA-differentiation therapy. Moreover, hypermethylated RAR beta can be used in combination with other hypermethylated markers for early detection of breast cancer on ductal breast cells obtained with procedures such as ductal lavage and nipple aspiration.

Therapeutic implications: The possibility to re-induce RAR beta activity in RA-resistant breast cancer cells, using both TSA and RA, a combination proven to be effective for treating leukemia (Grignani et al., 1998; Guidez et al., 1998; He et al., 1998; Lin et al., 1998; Warrell et al, 1998), is relevant to the treatment of RA-resistant breast tumors.

Preventive implications: DNA-methylation might be secondary to the inactive state of the RAR beta P2 promoter and might contribute to the extinction of RAR beta transcription. If we will prove that this hypothesis is true, RAR beta loss might be prevented in the presence either of supraphysiological levels of RA or other synthetic retinoids, or by retinoic acid metabolism blocking agents (RAMBAs) such as Liarazole (Miller, 1998).

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APPENDIX 1

Task 2

Copies of the following papers

1)) Evron E., Dooley WC, Umbricht CB, Rosenthal D, **Sacchi N**, Gabrielson E, Soito AB, Hung DT,Ljung BM, Davidson ME, Sukumar S Detection of breast cancer cells in ductal lavage fluid by methylation-specific PCR The Lancet 357, 1335-36, 2001

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Targeting epigenetic changes associated with retinoid resistance: a novel strategy to treat breast cancer
Am J Hum Genet Vol 69 (Supplement) A 151, p. 205, 2001

Research letters

Detection of breast cancer cells in ductal lavage fluid by methylation-specific PCR

Ella Evron, William C Dooley, Christopher B Umbricht, Dorothy Rosenthal, Nicoletta Sacchi, Edward Gabrielson, Angela B Soito, David T Hung, Britt-Marie Ljung, Nancy E Davidson, Saraswati Sukumar

If detected early, breast cancer is curable. We tested cells collected from the breast ducts by methylation-specific PCR (MSP). Methylated alleles of *Cyclin D2*, *RAR-β*, and *Twist* genes were frequently detected in fluid from mammary ducts containing endoscopically visualised carcinomas (17 cases of 20), and ductal carcinoma in situ (two of seven), but rarely in ductal lavage fluid from healthy ducts (five of 45). Two of the women with healthy mammograms whose ductal lavage fluid contained methylated markers and cytologically abnormal cells were subsequently diagnosed with breast cancer. Carrying out MSP in these fluid samples may provide a sensitive and powerful addition to mammographic screening for early detection of breast cancer.

The recent decline in breast cancer mortality rate is due, in part, to early diagnosis by screening mammography. However, given the well-recognised limitations of mammography,[1] further advances for early breast cancer detection are clearly needed.

We previously identified a number of genes that had lower expression in breast cancer than in healthy mammary epithelial cells using serial analysis of gene expression (SAGE) and microarray analysis of primary breast cancers. Many of these genes were silenced by hypermethylation of promoter sequences.[2,3] Sensitive methods of detection of methylated alleles have now enabled non-invasive detection of small numbers of cancer cells.[4] We searched for genes that were hypermethylated in more than 30% of breast cancers,[2,3] but unmethylated in healthy mammary epithelial cells, mammary stroma, and white blood cells. Three genes fulfilled this criteria: *Cyclin D2*,[2] *RAR-β*,[3] and *Twist* (Genbank accession number 003986). We found a cumulative incidence of methylation of the three genes in 48 (96%) of 50 surgically excised primary breast tumours and in eight (57%) of 14 of the ductal carcinoma in situ (DCIS) lesions. This analysis highlights the high sensitivity and specificity of a MSP-based test for breast cancer and raises the possibility that it could be applied to the detection of cancer cells in body fluids.

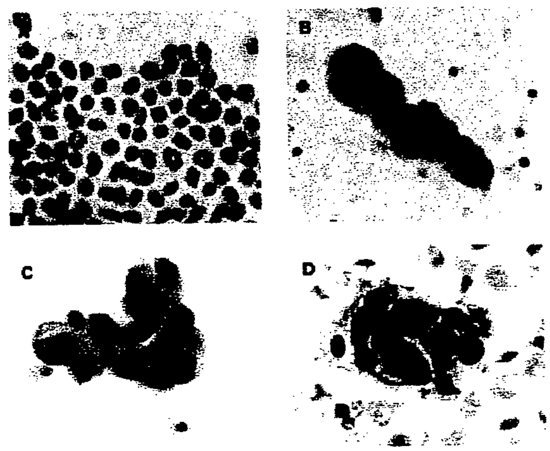

Figure 1: **Cytological analysis of ductal lavage fluid**
A: benign cells. B: atypical with mild changes. C: atypical with substantial changes. D: malignant cells.

Because most breast cancers arise from the ductal epithelium, atypical and malignant cells can be found in breast ductal fluid. We used two techniques to collect ductal fluid: Routine Operative Breast Endoscopy (ROBE) and ductal lavage. ROBE allowed direct visualisation of macroscopic changes in the ductal epithelium,[4] and recovery of irrigation fluid from the catheter. Ductal lavage through a micro-catheter; (Pro•Duct Health, CA), enabled collection of breast epithelial cells from the entire ductal tree. We cannulated the individual orifices with a small flexible microcatheter, and up

Diagnosis	*Cyclin D2*	*RAR-β*	*Twist*	Overall methylated*
Tissue	**n=140**	**n=140**	**n=140**	
Invasive breast cancer	25/50	17/50	21/50	48/50 (96%)
Ductal carcinoma in situ	4/14	7/14	4/14	8/14 (57%)
Normal breast tissue	0/20	0/20	0/20	0/20 (0%)
White blood cells	0/56	2/56	0/56	2/56 (4%)
ROBE fluid	**n=35**	**n=37**	**n=34**	
Invasive breast cancer	8/19	12/20	13/18	17/20 (85%)*
Ductal carcinoma in situ	2/6	1/7	0/7	2/7 (29%)
Atypical ductal hyperplasia	1/6	2/6	1/5	2/6 (33%)
No residual tumour	0/4	0/4	0/4	0/4 (0%)
Ductal lavage fluid	n=56	n=56	n=46	
Benign	3/45	2/45	0/35	5/45 (11%)
Atypical with mild changes	0/5	1/5	0/5	1/5 (20%)
Atypical with substantial changes	3/5	2/5	0/5	3/5 (60%)
Malignant	1/1	1/1	0/1	1/1 (100%)

ROBE=routine operative breast endoscopy

Assessing the use of methylation markers for early detection of breast cancer

*The number of overall methylated markers was significantly higher in malignant cases (invasive breast cancer and DCIS) than in non-malignant cases (healthy breast tissue, atypical ductal hyperplasias, and in samples from patients with no residual tumour; $p < 0.01$ by Pearson's χ^2). The number of overall methylated markers was significantly higher in cases classified as "atypical with marked changes" and "malignant", than in cases classified as "benign" and "atypical with mild changes" ($p < 0.01$ by Pearson's χ^2).

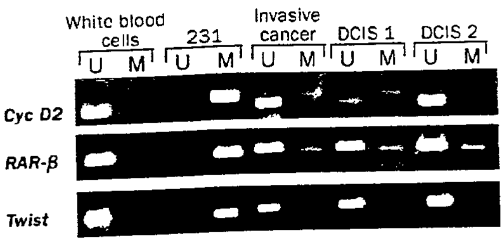

Figure 2: **MSP profiles of ductal lavage fluid**
U=unmethylated. M=methylated. 231=breast cancer cell line MDAMB 231. From women with invasive cancer and "malignant" cytology, and two women with ductal carcinoma in situ (DCIS1, DCIS2) and a cytology diagnosis of "atypical cells with marked changes". MSP primer sequences were: *RAR-β*: U Forward-5'GGATTGGGATGTTGAGAATGT3'; U Reverse-5'CAACC-AATCCAACCAAAACAA3'; M Forward 5'GAACGCGAGCGATTCGAGT3'; M Reverse-5'GACCAATCCAACCGAAACG3'. *Twist*: U Forward-5'TTTGGATGGG-GTTGTTATTGT3'; U Reverse 5'CCTAACCCAAACAACCAACC3' M Forward-5'TTTCGGATGGGGTTGTTATC3'; M Reverse-5'AAACGACCTAACCCGAACG3'. *Cyclin D2* analysis has been previously described.[2]

to 20 mL of saline was introduced in incremental volumes to flush out epithelial cells from the ducts and lobules. The ductal fluid was placed immediately in cytology fixative and prepared with standard millipore filtration devices for cytology assessment and DNA extraction.

We recruited 37 women with biopsy-proven cancer. Women underwent ROBE immediately before definitive surgery and after signing an informed consent form. DNA from both the ductal fluid cells and the matching surgical samples was tested with methylation-specific PCR (MSP) for *Cyclin D2*, *RAR-β*, and *Twist*.[2,3]

Methylated alleles of at least one of three markers were detected in 17 of 20 irrigation fluid samples from patients with pathology-confirmed invasive carcinoma (table). Healthy breast tissue contained only unmethylated genes (zero samples of 20; table). Methylated alleles for *RAR-β* only were noted in two of 56 samples (table).

By contrast, irrigation fluid from four patients who underwent re-excision, but were subsequently found to be tumour-free, contained only unmethylated markers (table). Irrigation fluid from two of seven patients with DCIS (Grade 1-3), and two of six patients with atypical ductal hyperplasias contained hypermethylated markers. DNA samples from 19 of the 20 excised tumour samples were positive by MSP for the presence of methylated markers. Analysis of the irrigation fluid thus missed two MSP-positive samples, presumably because of the low cell yields. Cytology analysis on this fluid was inconclusive in 23 samples due to inadequate cellularity, and no malignant cells were detected in the remaining samples. These results suggest that MSP is sensitive, as the techinique detected cancer cells in 85% of ductal fluid samples from patients with breast malignancy, including cases where the material was inadequate for cytology.

We extended our analysis to 56 samples of ductal lavage fluid (obtained after informed consent) from women with non-suspicious mammograms and breast examinations, but at high risk for developing breast cancer (as defined by a Gail index $\geq 1{\cdot}7$, previous history of contralateral breast cancer, or *BRCA1* and *BRCA2* mutations). Using cytopathology, 50 samples were classified as benign or with mild changes, and six samples were classified as atypical with substantial changes or frankly malignant (figure 1). Among the cases with substantially abnormal cells or malignant cells, four of six samples were identified by MSP (67% sensitivity), whereas only five of 45 benign cases were positive (89% specificity; figure 2). Pathologically confirmed breast cancer was subsequently diagnosed in two women with abnormal cytological findings and MSP-positive ductal lavage fluid. A third patient in this category is undergoing further assessment. These cases indicate the promising potential of the MSP-based method for early detection of breast malignancy, before the appearance of suspicious findings on mammography.

MSP confirmed the cytological finding that led to the diagnosis of breast cancer in two women. In combination with cytology evaluation, MSP of ductal lavage could provide a useful adjunct to mammography in the early diagnosis of breast cancer.

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MUC 1: a genetic susceptibility to infertility?

Andrew W Horne, John O White, Raul A Margara, Ross Williams, Robert M L Winston, El-Nasir Lalani

In man and some animals regulation of embryo implantation by endometrial expression of the highly polymorphic MUC 1 mucin has been suggested. We assessed the polymorphism of *MUC 1* in women known to be fertile and those with infertility due to suspected failure of embryo implantation. The median of the lower allele size in the infertile group was only 2·5 kb compared with 3·4 kb in the fertile group (p=0·0029, difference 0·9, [95% CI 0·1–1·3]). Women with unexplained infertility might have a genetic susceptibility to failure of embryo implantation due to small *MUC 1* allele size.

Despite thorough investigation many cases of infertility remain unexplained. Although morphologically normal embryos are transferred to the uterus in most in-vitro fertilisation (IVF) cycles, successful pregnancy only takes place in about one in five attempts. Since at least 50% of IVF embryos develop to the blastocyst stage in culture, failure of implantation is probably the reason for failure of treatment.[1]

The essential cellular factors in endometrium that contribute to implantation are not fully understood. MUC 1 mucin, an oxygen-glycosylated (O) epithelial glycoprotein, could potentially modulate embryo attachment. It extends beyond the endometrial glycocalyx and is probably the first molecule that the embryo encounters on attachment.[2]

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ings from 3 of 3 (100%) patients without CIN, in 8 of 8 (100%) with CIN I, 9 of 9 (100%) with CIN II, 7 of 10 (70%) with CIN III, and 4 of 4 (100%) with cervical cancer. hTERT mRNA was detected in scrapings from 0 of 3 (0%) patients without CIN, in 1 of 8 (13%) with CIN I, 1 of 9 (11%) with CIN II, 1 of 9 (11%) with CIN III, and 3 of 3 (100%) with cervical cancer ($p<0.05$). The sensitivity and negative predictive value of the hTERT mRNA rt-PCR for CIN II or higher lesions were 24% and 40%, respectively, while specificity for no CIN or CIN I was 91%. Conclusion: These preliminary results show that hTERT mRNA is more frequently observed in higher grade CIN/cervical cancer scrapings, while hTR is detectable in almost every scraping. hTERT mRNA rt-PCR assessment in cervical scrapings has a low sensitivity for CIN II or higher and therefore does not appear to be useful in primary screening for (pre)neoplastic cervical disease. A more extended serie of patients will be presented. This study was supported by grant NKB/KWF 97-1581.

#1346 Detection and Clinical Application of Serum Free Telomeric Fragments (FTF) in Ovarian Cancer. Tomoko Idei, K Ohtani, H Sakamoto, A Kikuchi, M Hattori, T Takami, and T Yamamoto. *Nihon Univ.Faculty of Med., Tokyo, Japan.*

Objective: To detect free telomeric fragments (FTF) from serum DNA of ovarian cancer patients and determine their clinical application. Methods: A blood sample was obtained from 143 ovarian cancer patients at before and after chemotherapy. Control serum was obtained from 52 healthy women. Genomic DNA was extracted from the filtrated serum and FTF were amplified by PCR using a modified telomeric repeat amplification protocol assay. The amplified DNA fragments were separated by 0.7% agarose gel electrophoresis and autoradiographed. The band intensity was analyzed with NIH Image software. Results: Three major FTF of 23-30, 9.5-9.8 and <6 kB were detected in the serum from both cancer patients and control women. The change of 9.5-9.8 kB FTF density was associated with the response to chemotherapy. Conclusion: 9.5-9.8 kB FTF was detected in the serum DNA of ovarian cancer patients and has a possible role on evaluation of response to chemotherapy. The change of FTF length could be an earlier maker for drug resistance than tumor marker.

#1347 Resistance of Primary Cultured Mouse Hepatic Tumor Cells to Cellular Senescence Despite Expression of $p16^{INK4a}$, $p19^{ARF}$, p53 and $p21^{WAF1}$. Masahiko Obata, Emi Imamura, Yukinori Yoshida, Junichi Goto, Kan Kishibe, Atsumi Yasuda, Yuji Yaginuma, and Katsuhiro Ogawa. *Asahikawa Medical College, Asahikawa, Japan.*

Primary cultured normal mouse hepatic cells became senescent within a short period, although rare cells formed colonies from which continuously proliferating cell lines could be established. In contrast, the hepatic tumor (HT) cells showed little senescence, less decline in cell number and higher colony forming capacity.This property may be either due to defect in the senescence program or gain in some override function . Although the molecular mechanisms that regulate senescence are not well understood, at least two signaling pathways have been suggested to be important. One of them is the $p16^{Ink4a}$ -Rb pathway. Overexpression of $p16^{Ink4a}$ then seems to be linked to the senescence phenotype, while its loss may result in immortalization. Another important signaling pathway controlling senescence is the $p19^{Arf}$-p53 pathway. Because *$p19^{Arf}$* -knockout mouse embryonic fibroblasts continue to divide, *$p19^{Arf}$* also has a critical role in senescence. To assess the basis for the difference in senescence property between normal mouse hepatic cells and HT cells, we investigated the expression of $p16^{Ink4a}$, $p19^{Arf}$, p53 and $p21^{Waf1}$ genes related to senescence/immortalization, in the primary normal and HT cells, together with cell lines established from both. In the primary normal cells, although $p16^{Ink4a}$ and $p19^{Arf}$ were not expressed during the early phase, they became up-regulated in association with senescence, but disappeared in later stages of colony formation. In contrast, the primary HT cells expressed both $p16^{Ink4a}$ /$p19^{Arf}$ from the beginning, with sustained expression during cultivation. No alterations of $p16^{Ink4a}$ /$p19^{Arf}$ such as deletion, mutations or hypermethylation were detected in the primary HT cells, although most cell lines derived either from normal liver or HT cell colonies lost the $p16^{Ink4a}$ and/or $p19^{Arf}$ expression due to hypermethylation or homozygous deletion at the *Ink4a/Arf* locus. On the other hand, the primary normal and HT cells and all but one of the cell lines showed constitutively-elevated expression of p53 and $p21^{Waf1}$, with a further increment in association with growth arrest after UV radiation, indicating a functionally normal p53 pathway. These results indicate that primary HT cells are resistant to senescence despite retaining $p16^{Ink4a}$, $p19^{Arf}$, p53 and $p21^{Waf1}$expression, while both normal and HT cell lines frequently show loss of $p16^{Ink4a}$ /$p19^{Arf}$ function.

#1348 Decreased Levels of Adenine Nucleotide Translocase and Functional Changes of the Permeability Transition Pore Complex in Mitochondria of Senescent Cells. Mary M. Sugrue and Yan Wang. *Mount Sinai School of Medicine, New York, NY.*

Decreased mitochondrial membrane potential has been found in a variety of cells undergoing cellular senescence/aging. Cells undergoing apoptosis also exhibit decreased mitochondrial membrane potential, which has been shown to result from activation of the mitochondrial permeability transition pore complex (PTPC). At present we do not know the mechanism regulating mitochondrial membrane potential in cell senescence. Previously, we showed that decreased mitochondrial membrane potential in senescent cells is associated with decreased responsiveness of the mitochondrial PTPC to pharmacologic agents that induce its closure and inhibit the pore (e.g. cyclosporin A). We hypothesize that decreased mitochondrial membrane potential in senescent cells is characterized by distinct structural/functional changes in the PTPC. We analyzed the protein levels of adenine nucleotide translocase (ANT), a major component of the PTPC, in our tet-inducible EJ-p53 cells and in IMR-90 fibroblasts using an ANT-specific polyclonal antiserum and immunoblot analysis. The level of ANT was significantly decreased in senescent EJ-p53 cells compared to controls. Similarly, ANT was decreased in senescent vs. young IMR-90 fibroblasts. Using laser scanning confocal microscopy, we examined the level and subcellular localization of ANT in senescent cells. We found that ANT is decreased in mitochondria of senescent cells compared to controls. Moreover, in senescent cells the decreased ANT co-localized to mitochondria exhibiting significantly decreased mitochondrial membrane potential. To examine the functional significance of decreased ANT in senescent cells, we analyzed their response to atractyloside (ATR), an agent that binds ANT and activates (opens) the PTPC. Mitochondrial membrane potential did not decrease significantly nor was there evidence of apoptosis in senescent cells treated with ATR in contrast to controls, indicating that senescent cells exhibited decreased responsiveness to ATR. Together these results indicate that senescent cells are characterized by decreased levels of ANT, the major component of the PTPC, which may induce specific changes in the structure/function of the PTPC, and mediate the decreased mitochondrial membrane potential unassociated with apoptosis in addition to the decreased responsiveness to agents that activate or inhibit the PTPC. Furthermore, decreased ANT and the concomitant changes in the PTPC may contribute to the resistance to apoptosis that characterizes senescent cells.

#1349 Acetylation Sites Govern PKA-Dependent Androgen Receptor Function. Maofu Fu, Jian Wang, Chenguang Wang, and Richard Pestell. *Albert Einstein College of Medicine, New York, NY.*

The androgen receptor (AR) is a member of a nuclear receptor superfamily which bind specific DNA sequences and regulates ligand-dependent gene transcription. The activation of the androgen receptor (AR) by dihydrotestosterone (DHT) plays a key role the development of male secondary sexual characteristics and may contribute to prostate cancer onset and/or progression. Protein kinase A stimulates dephosphorylation of the AR inhibiting AR activity. In recent studies, the AR was shown to serve as a substrate for acetylation by the histone acetylases, p300 and P/CAF. Through mass spectrometry and edman degradation analysis, acetylation of the AR was shown to occur at a highly conserved lysine rich motif. Although the AR undergoes modification by phosphorylation and acetylation, the relationship between these two post translational events is unknown. In order to understand the functional relationship between phosphorylation and direct acetylation of the AR, a point mutation of the AR was constructed at the site of preferential in vitro acetylation ($AR_{K(630)A}$) and activity assessed in vivo. DHT-induced AR activity was assessed using reporter gene assays in prostate cancer cell lines. Compared with wild type AR, mutation of AR lysine 630 abolished DHT-induced activity. Induction by the co-activator p300 in the presence of ligand was also abolished by the acetylation mutation. To examine the role of endogenous acetylation/deacetylation, the effect of the histone deacetylase inhibitor trichostatin A (TSA) was assessed. In contrast with the wild type AR, which was induced in a dose-dependent manner by TSA, the $AR_{K(630)A}$ mutant was not activated by TSA. As cAMP has been shown to inhibit liganded AR-activity, we assessed the function of the AR acetylation site in cAMP-regulated activity. Forskolin treatment or co-expression of the PKAc subunit, inhibited liganded ARwt activity 50%, and the addition of the PKA inhibitor H89 (30 nM) augmented DHT-induced activity of the ARwt 2.5-fold. In contrast neither agent affected activity of either ($AR_{K(630)A}$) or ($AR_{KK(632/633)AA}$) a mutant of the additional two AR acetylation sites. In contrast with the wild type AR, which exhibits multiple phosphorylation-dependent species, the acetylation mutants lack phosphorylated forms by electrophoretic mobility. Together these studies suggest the conserved AR lysine motif that functions in acetylation by co-activators, determines both DHT and cAMP regulated function.

#1350 Chromatin Remodeling at RAR β Promoter 2 Is Associated with RA-Resistance in Breast Cancer. Nicoletta Sacchi, Silvia Sirchia, Elena Sironi, and Saraswati Sukumar. *Johns Hopkins University, Baltimore, MD, and School of Medicine, Milan, Italy.*

The expression of the retinoic acid receptor beta, RAR beta, one of the nuclear receptors that mediates retinoic acid (RA) activity is found severely downregulated, or lost, in breast cancer cells. We and others, have observed that DNA methylation can affect one of the two RAR beta promoters, RAR beta 2, in both breast cancer cell lines and tumors. Treatment of cells with a methylated promoter by means of the DNA methyltransferase inhibitor 5-Aza-2' deoxycytidine led to demethylation of the RAR beta 2 promoter and expression of RAR beta indicating that DNA methylation is at least one factor, contributing to RAR beta inactivity. However, we found that in RA-resistant breast cancer cell lines with a methylated RAR beta 2 promoter, RA-induced RAR beta transcription, can be restored from a methylated promoter by the histone deacetylase (HDAC) inhibitor trichostatin A (TSA). Thus, an appropriate level of histone acetylation of the chromatin embedding RAR beta 2 seems to be a condition necessary and sufficient for the expression of RAR beta, even in the presence of methylation. This conclusion is supported by our findings showing the relationship between DNA methylation and histone acetylation of RAR beta 2 in both RA-inducible and

RA-resistant breast cancer cell lines. From our data we conclude that chromatin remodeling, due to an altered acetylation state at RAR beta 2, is crucial in the development of RA-resistance in breast cancer cells and that a combination of HDAC inhibitors and RA can be beneficial to restore RA sensitivity in RA- resistant breast tumors.

#1351 Map Kinases Mediate UVB-Induced Phosphorylation of Histone H3 at Serine 28. Shu-ping Zhong, Hidemasa Goto, Masaki Inagaki, and Zigang Dong. *Aichi Cancer Center Research Institute, Aichi, Japan, and The Hormel Institute, Austin, MN.*

Histone H3 phosphorylation is closely related to chromatin remodeling and chromosome condensation. H3 phosphorylation at serine 28 is coupled with mitotic chromosome condensation in diverse mammalian cell lines. However, the pathway that mediates phosphorylation of H3 at serine 28 is not known. In the present study, ERK1, ERK2, and p38 kinase strongly, but JNK2 and JNK1 slightly, phosphorylated H3 serine 28 *in vitro*. UVB irradiation markedly induced phosphorylation of H3 at serine 28 in JB6 C1 41 cells. PD 98059, a MEK1 inhibitor, and SB 202190, a p38 kinase inhibitor, efficiently inhibited UVB-induced H3 phosphorylation at serine 28. Expression of dominant negative mutant (DNM) ERK2 in JB6 C1 41 cells totally, while DNM p38 kinase or JNK1 partially, blocked UVB-induced phosphorylation of H3 at serine 28. Further, UVB-induced H3 phosphorylation at serine 28 was inhibited in *Jnk1*$^{-/-}$ cells, but not in *Jnk2*$^{-/-}$ cells. These results suggest that UVB-induced H3 phosphorylation at serine 28 is mediated by MAP kinases. [Supported by National Institute of Health grant CA77646 and The Hormel Foundation.]

#1352 Deregulated Polyamine Biosynthesis Alters Histone Acetylation in Murine Skin and Tumors. Cheryl A. Hobbs, Barry A. Paul, Phuong My Chung, and Susan K. Gilmour. *Lankenau Institute for Medical Research, Wynnewood, PA, and Rosemont College, Rosemont, PA.*

Elevated expression of ornithine decarboxylase (ODC), an enzyme which catalyzes the first step in the biosynthesis of polyamines, is characteristic of many solid tumors. The crucial role of polyamines in regulating cell proliferation and differentiation may be partially attributed to their influence on gene expression. Dynamic changes in the acetylation state of nucleosomal histones are critical for regulating the transcriptional competency of chromatin, but effects of polyamines on histone acetylation *in vivo* have never been demonstrated. We used the K6/ODC and ODC/Ras transgenic mouse models, in which constitutive expression of ODC is targeted to hair follicles, to examine the effects of elevated levels of polyamines on histone acetylation in epithelial cells in skin. As compared to normal littermate controls, overall acetylation of histones is reduced in ODC transgenic skin and in the skin tumors which develop spontaneously in ODC/Ras double transgenic mice. Although intrinsic histone deacetylase (HDAC) activity typically is elevated in ODC transgenic skin, it is generally reduced in ODC/Ras tumors, and therefore most likely doesn't account for the decreased acetylation of histones observed in tumors. Intrinsic histone acetyltransferase (HAT) activity is also elevated in homogenates of ODC transgenic skin and is exceptionally high in the ODC/Ras tumors. The HAT activity present in ODC transgenic skin and tumor tissue is predominately directed at histone H4 and is characterized by a distinct substrate preference for lysine-12 in the H4 tail domain. Significantly, treatment with the ODC inhibitor α-difluoromethylornithine (DFMO), which results in regression of ODC/Ras tumors, restores acetylation to normal levels in cultured K6/ODC keratinocytes, and increases acetylation of histones in K6/ODC skin and in regressing tumors and nontumor-bearing skin of ODC/Ras mice. DFMO treatment also abrogates some of the enhancement effects on intrinsic HAT and HDAC activities observed in ODC/Ras skin and tumors. Thus, the altered HAT and HDAC activities and histone acetylation in ODC transgenic keratinocytes and skin are linked to the high intracellular pools of polyamines. In addition, immunoblot analyses using various antibodies directed against acetylated lysine residues suggest altered acetylation of several non-histone proteins, including p53, in ODC transgenic skin. Our results indicate that multiple mechanisms exist by which polyamines may both positively and negatively influence histone acetylation in mammals. Furthermore, these studies imply that the aberrant polyamine biosynthesis characteristic of many solid tumors leads to abnormal chromatin remodeling, potentially contributing to altered gene expression and cellular transformation. (Funded by NIH CA 75756 and CA 70739)

#1354 Chromatin Structure Opening by the Histone Deacetylase Inhibitor Trichostatin A (TSA) Increases Cellular Cytotoxicity to Topoisomerase Inhibitors. France Carrier, Mellissa Blake, and Tayeb Khelifa. *University of Maryland, Baltimore, Baltimore, MD.*

Inhibitors against the Topoisomerase (Top) enzymes are widely used in most chemotherapeutic regimens. Since the Top inhibitors are particularly efficient during the S-phase of the cell cycle, it is thought that ongoing DNA synthesis is involved in the cellular killing by these agents. Nonetheless, it is also possible that the looser chromatin structure encountered during the S-phase of the cell cycle facilitates accessibility to DNA and consequently the trapping of the Top-DNA complexes by the inhibitors. Our preliminary data have shown that cell lines expressing reduced levels of the acidic protein Gadd45 are more resistant to Top inhibitors. Gadd45 can facilitate Top relaxing and cleavage activity in the presence of core histone. Moreover, Gadd45 can bind to hyperacetylated mononucleosome and increase Top binding to mononucleosomes. Gadd45 could thus stabilize a more open chromatin structure and facilitate DNA accessibility to the Top enzymes. To ascertain whether a more open chromatin stucture could actually affect the efficiency of Top inhibitors, we have treated the human colon carcinoma RKO cells with the histone deacetylase inhibitor TSA for 4h. The cells were then washed and exposed to increasing amount (0-10 μM) of VP-16, a Top2 inhibitor for 4h. Survival analyses performed two weeks latter indicate that pretreatment of the cells with TSA increases the capacity of VP-16 to kill cells by five fold. Higher doses of VP-16 and different time exposure to TSA are currently under study. The effects of TSA on D54, HBT-20 and HBT-28, three human brain tumor cell lines clinically resistant to Top2 inhibitors, are also under study. These data suggest that chromatin opening by either acidic proteins or histone deacetylase inhibitors could have clinical significance especially in slow growing tumors resistant to Top inhibitors.

#1355 Ultraviolet Light Transiently Increases Histone Acetylation That Coincides with Transcriptional Activation of Human Immunodeficiency Virus. Sharon K. Bullock, Mohiuddin Taher, Kate Sleeth, Jeffrey Alexander, and Kristoffer Valerie. *VCU, Richmond, VA.*

Modulation of chromatin structure is thought to regulate in part processes associated with DNA, such as transcription, replication, and repair. This premise assumes that decondensation of chromatin allows the appropriate factors better access to the DNA. It is widely accepted that changes in chromatin structure that facilitate transcriptional activation involve acetylation of lysine residues of core histones associated with promoter regions. Treatment of cells with inhibitors of histone deacetylases results in increased global histone acetylation and increased transcription from the human immunodeficiency virus (HIV) promoter that coincides with increased promoter accessibility. Here we report the results from studies geared towards elucidating steps involved in UV activation of HIV-directed gene expression and the notion that histone acetylation may be involved in this process. Western analyses of whole cell lysates demonstrated that UV irradiation of HIVcat/HeLa cells produced both time (peak at 5 min) and dose-dependent increases in histone acetylation (up to 4-fold). As expected, treatment of cells with trichostatin A (TSA), an inhibitor of histone deacetylases (HDAC), increased acetylation of histone H4. Extracts from UV- and TSA-treated HIVcat/HeLa cells demonstrated increased CAT activity to a similar extent. To determine whether increased histone acetylation occurred on the HIVcat transcription unit, we performed chromatin immunoprecipitation (CHIP) assays on UV- and TSA-treated HIVcat/HeLa cells using anti-acetyl histone antiserum. PCR analysis revealed increased presence of HIVcat DNA in the immunoprecipitate in a time-dependent fashion to ~1.7-fold at 10 min that then subsided to below basal levels at 60 min. On the other hand, TSA treatment resulted in a maximum ~6-fold increase at 24 h (2-fold at 60 min). Taken together, the data suggests that UV-induced activation of HIV gene expression could be mediated at least in part by increased histone acetylation interacting with the HIV promoter. Supported by CA53199 (KV).

#1356 Activation of Chromatin Structure on the Up-Regulated Genes in Cisplatin Resistant Cells. Minoru Nomoto, Gunnji Nagatani, Ken Kato, Hiroto Izumi, and Kimitoshi Kohno. *1st Dept. Int. Med., Kyushu Univ., Fukuoka, Japan, and Univ. of Occupational & Environmental Health, Japan, Kitakyushu, Fukuoka, Japan.*

In order to analyze in vivo chromatin structure on the desired regions of particular genes, we used Ligation-mediated PCR. Genes of transcription factor YB 1 and Stat 3, DNA repair associated HMG 1 and ERCC 1 were up-regulated in cisplatin resistant cells. Marked differences of transcription factors binding in promoter regions of these genes were not observed between parental cells and resistant cells from the analysis of in vivo Footprint experiment. However, micrococcal nuclease (MNase) cleavage sites in resistant cells were distributed even in nucleosome position, whereas cleavage signals were restricted in linker portion in parental cells. These cleavage signals in resistant cells were obtained at much lower dose points of MNase comparing with those in parental cells. Thus, the nucleosomes on promoter regions of up-regulated genes are activated. Acetylation status of histones on the activated nucleosome are now under investigation.

#1357 Regulation of Histone Acetylation and Erythroleukemia Cell Growth by Some Allyl Compounds. Michael A. Lea, Mariam Rasheed, and Verrell M. Randolph. *UMDNJ - New Jersey Medical School, Newark, NJ.*

Since previous work indicated that some allyl sulfur compounds can inhibit cancer cell growth and increase the acetylation of histones we have extended these observations to additional allyl derivatives. S-allyl cysteine and S-allylmercaptocysteine were provided by Wakunaga of America Co., Ltd., and their action was compared with that of allyl phenyl sulfone and allyl butyrate. Growth of DS19 mouse erythroleukemia cells was decreased by 5mM S-allyl cysteine and 1mM allyl phenyl sulfone while 5mM allyl phenyl sulfone blocked cell proliferation and resulted in cell death. 1mM allyl butyrate had a small effect on cell growth while 2.5mM allyl butyrate killed the cells. S-allylmercaptocysteine caused a significant inhibition of growth at 0.5μM and blocked cell proliferation above 2μM. S-allyl cysteine and allyl phenyl sulfone had negligible effects on histone deacetylase and S-allylmercaptocysteine, at concentrations up to 1mM, had only a minor effect on the activity of this enzyme. Allyl butyrate inhibited histone deacetylase at lower concentrations than the other three compounds but was less effective than S-allylmercaptocysteine for the induction of histone acetylation. Acetylation of H4

Rec.

MOLECULAR DETECTION OF BREAST CANCER CELLS IN DUCTAL LAVAGE FLUID (DLF) USING METHYLATION SPECIFIC PCR. Ella Evron, William Dooley, Christopher Umbricht, Dorothy Rosenthal, Nicoletta Sacchi, Nancy Davidson, Saraswati Sukumar, Departments of Oncology, Surgery, and Pathology, Johns Hopkins University School of Medicine, Baltimore, MD.

Breast cancers originate from the epithelial cells lining the breast ducts, and all ducts converge to 5-8 openings at the nipple. Recently, a technique for ductal visualization and lavage through a microcatheter has been developed, enabling imaging and aspiration of fluid from single breast ducts. Since breast cancers arise from the ductal epithelium, it is likely that malignant cells may be represented in the ductal fluid. Promoter hypermethylation of specific genes is increasingly recognized as a cancer specific phenomenon. Based on that, we developed a detection assay for breast cancer in DLF. First, we assembled a panel of genes, Cyclin D2, Twist, RAR-β2, and estrogen receptor (ER), which had a 96% (48/50) cumulative incidence of promoter hypermethylation in surgically excised primary breast tumors, as tested by methylation specific PCR (MSP). Normal breast tissue (n=20) and peripheral blood cells (n=10) contained only unmethylated gene-promoter sequences. Using this panel, we analyzed DLF from patients with benign or malignant tumors of the breast, for the presence of methylated alleles of these genes. The fluid was obtained during an endoscopic visualization procedure called ROBE. Surgically excised tumors from the same patients were also subjected to MSP analysis for these markers. Only unmethylated DNA was detected in 5 of the samples obtained from tumor free ducts (n=1) and ducts containing benign lesions (n=4). Hypermethylated DNA was detected in 10 of 13 (76%) ductal fluid samples, all from patients with invasive breast cancer. 12 of 13 (92%) of the surgically excised tumors contained hypermethylated markers. Thus, our assay detected tumor cells in 10/12 potentially positive DLF samples. Detection of promoter hypermethylation by MSP is a powerful tool for diagnosis of breast cancer in DLF. Further evaluation of this diagnostic method is warranted.

Tissue	Cyclin D2	RAR-beta2	Twist	ER	Overall Methylated
Breast Ca.	25/50	17/50	21/50	15/50	48/50
Normalbreast	0/18	0/18	0/18	0/18	0/18
WBC	0/10	0/10	0/10	0/10	0/10
DLF-Inv. Ca	4/13	5/13	6/10	1/3	10/13
DLF-Benign	0/5	0/5	0/5	-	0/5

(No. Methylated / Total No. Tested)

RAR beta gene re-expression therapy for breast cancer

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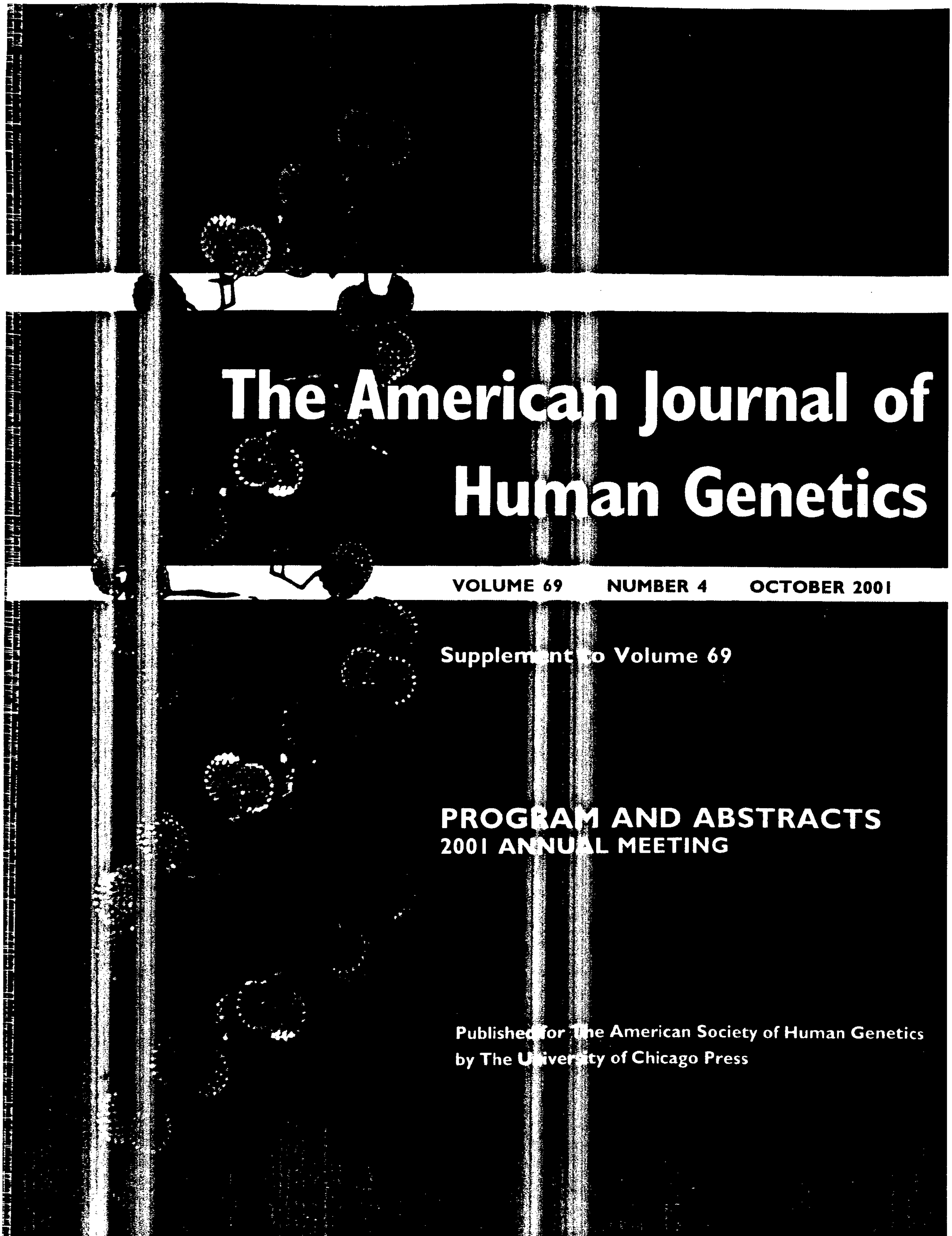
The expression of the retinoic acid receptor beta, RAR beta, one of the nuclear receptors that mediates retinoic acid (RA) activity is found severely downregulated, or lost, in breast cancer cells. We have observed that DNA methylation at one of the two RAR beta promoters, RAR beta P2, in both breast cancer cell lines and primary tumors is associated with RA-resistance. Our data clearly show that: 1) in breast cancer cell lines with an unmethylated P2, re-acetylation at P2 is achieved by RA treatment alone and 2) in breast cancer cell lines with a methylated P2, RAR beta transcription can be restored only by using histone deacetylase inhibitors (HDACIs), such as trichostatin A (TSA) and phenyl butyrate (PB), in association with RA. Thus , histone re-acetylation of the chromatin embedding RAR beta P2 seems to be an absolute requirement for restoring the expression of RAR beta in the presence of RA. Apparently, RAR beta transcription can be restored even if P2 remains methylated, suggesting that RAR beta re-expression is possible when RAR P2 undergoes an appropriate chromatin remodeling consequent to reacetylation of the promoter region. We conclude that: 1) the RAR beta P2 methylation status represents an useful "predictor" of RA response in breast cancer and 2) an appropriate combination of HDACIs and RA can be beneficial to restore RA sensitivity in RA-resistant breast tumors with a methylated P2 .



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